

Assessment of anti-piperacillin IgG binding to structurally-related drug protein adducts

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ABSTRACT: The risk of developing hypersensitivity to alternative antibiotics is a concern for penicillin hypersensitive patients and healthcare providers. Herein we use piperacillin hypersensitivity as a model to explore the reactivity of drug-specific IgG against alternative β -lactam protein adducts. Mass spectrometry was used to show the drugs (amoxicillin, flucloxacillin, benzyl penicillin, aztreonam and piperacillin) bind to similar lysine residues on the protein carrier bovine serum albumin. However, the hapten-specific IgG antibodies found in piperacillin hypersensitive patient plasma did not bind to other β -lactam protein conjugates. These data outline the fine specificity of piperacillin-specific IgG antibodies that circulate in patients with hypersensitivity.

β -lactam antibiotics contain a β -lactam ring structure that is critical for their anti-bacterial activity. These drugs are used either alone or in combination with β -lactamase inhibitors which serve to conserve pharmacological activity and extend their spectrum. β -lactam antibiotics have been implicated in a plethora of cutaneous hypersensitivity reactions that vary in severity and pathophysiology. Since all β -lactam antibiotics contain the same reactive strained ring structure that is susceptible to nucleophilic attack by lysine residues on protein it is likely that similar neo-epitopes are generated in patients. Thus, inadvertent activation of the adaptive immune system in hypersensitive patients due to cross-reactivity is an important clinical concern.

Piperacillin is a β -lactam antibiotic commonly used to combat respiratory tract infections in patients with cystic fibrosis. Hypersensitivity reactions occur at a higher frequency in patients with cystic fibrosis when compared to the general population with an incidence of up to 30 % reported in some studies.¹ Thus, piperacillin hypersensitivity represents a relevant model to study the role of the adaptive immune system in the disease pathogenesis. We have recently found that T-cells from hypersensitive patients are activated with piperacillin albumin adducts following protein processing by antigen presenting cells.^{2,4} T-cell cross-reactivity with different drugs was not observed.⁵ In recent studies we have also detected piperacillin hapten-specific B-cells in the peripheral blood of hypersensitive patients.⁶ Stimulation of the B-cells *in vitro* resulted in the secretion of IgG antibodies and the same antibodies were found to circulate in patient plasma.⁶ In the current study, structurally diverse β -lactam bovine serum albumin (BSA) adducts were synthesized, characterized and used to explore whether IgG antibodies from patients react with different β -lactam structures (Figure 1). BSA

was selected as a protein carrier since it contains a similar number of lysine residues to human serum albumin – the major target for piperacillin binding in patients – and the native protein displays limited reactivity against piperacillin-specific IgG.⁶

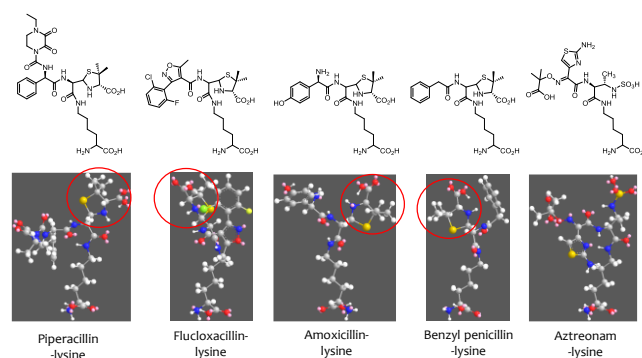


Figure 1. Structure of the β -lactam haptens bound to lysine residues on BSA. Red circles show the thiazolidine ring.

Plasma from four hypersensitive patients was used to study IgG binding. Each patient developed maculopapular exanthema with or without drug-induced fever 3-7 days after initiation of piperacillin therapy. Blood samples were obtained with written consent and after approval by the Leeds East Ethics Committee. Peripheral blood mononuclear cells (PBMCs) were isolated using ficoll density centrifugation. The clinical diagnosis of piperacillin hypersensitivity was confirmed by *in vitro* assessment of T-cell proliferative responses using the lymphocyte transformation test. Lymphocytes from all four patients were stimulated to proliferate with piperacillin in a concentration-dependent manner (results not shown).

To prepare adducts, drugs (amoxicillin, flucloxacillin, benzyl penicillin, aztreonam and piperacillin) were incubated with BSA for 96 h at a protein-drug molar ratio of 1:50. Ice-cold methanol was used to precipitate protein and the Bradford assay was used for protein quantification. Protein conjugates were purified using centrifugal filters with the 3KDa cut-off. Western blotting using anti-penicillin or anti-flucloxacillin antibodies was performed to confirm β -lactam-protein binding; visible bands at 66kDa were observed for all drug protein adducts. Adducts were digested with trypsin prior to mass spectrometric analysis of site of drug modification. Samples were reconstituted in 2% acetonitrile / 0.1% formic acid (v/v) and delivered into a QTRAP 5500 hybrid quadrupole-linear ion trap mass spectrometer (Sciex) by automated in-line LC using an established method.³ MRM transitions specific for drug-modified peptides were selected as follows: the mass/charge ratio (m/z) values were calculated for all possible peptides with a missed cleavage at a lysine residue; to these were added the mass of the appropriate hapten; the parent ion masses were then paired with relevant fragment masses. MRM transitions were acquired at unit resolution in both the Q1 and Q3 quadrupoles to maximize specificity. They were optimized for collision energy and collision cell exit potential, and the dwell time was 20ms. MRM survey scans were used to trigger enhanced product ion MS/MS scans of drug-modified peptides, with Q1 set to unit resolution, dynamic fill selected, and dynamic exclusion for 20s. Total ion counts were determined from a second aliquot of each sample analysed by conventional LC tandem MS and were used to normalize sample loading on column. MRM peak areas were determined by MultiQuant 1.2 software (Sciex). Epitope profiles were constructed by comparing the relative intensity of MRM peaks for each of the modified lysine residues within a sample and normalization of those signals across samples. Epitope profiles showed that a similar panel of lysine residues on BSA was modified with amoxicillin, benzyl penicillin, piperacillin and flucloxacillin (Figure 2). Lysine residues on all 4 conjugates were haptenated at positions 4, 12, 211, 431 and 524. Three of the drugs modified lysine residues at positions 132, 136 and 523. Amoxicillin and benzyl penicillin alone modified lysine 20, 473 and 537, while lysine 535 was only modified with flucloxacillin. A more restricted binding profile was observed with aztreonam; modifications were only detected on lysine residues at positions 136, 211 and 524.

ELISA was used for the detection of anti-drug IgG antibody levels in hypersensitive patient plasma. Plates were coated overnight at 4 °C with a 20 μ g/ml solution of drug BSA adducts. Piperacillin (1 mM) was added to certain wells for 16 h, prior to the addition of BSA adducts, for hapten inhibition. Following washing and blocking, a 1/10 dilution of plasma from the patients was added. Goat anti-human IgG, a horseradish peroxidase-labelled rabbit anti-goat IgG and TMB substrate were used to visualize bound anti-piperacillin IgG. Absorbance values were read at 490nm using an automated plate reader (Dynatech MR600, Hendersonville, TN, USA).

Substantial piperacillin IgG binding to piperacillin-modified-BSA was observed with plasma from all four patients (Figure 3). Antibody binding was reduced by greater than 85% when the antibody was pre-incubated with soluble piperacillin in hapten inhibition experiments. Antibody binding was not observed when the piperacillin-BSA adduct was replaced with amoxicillin, aztreonam, or benzyl penicillin-BSA adducts, while very low levels of binding was detected with the flucloxacillin-BSA adduct (Figure 3).

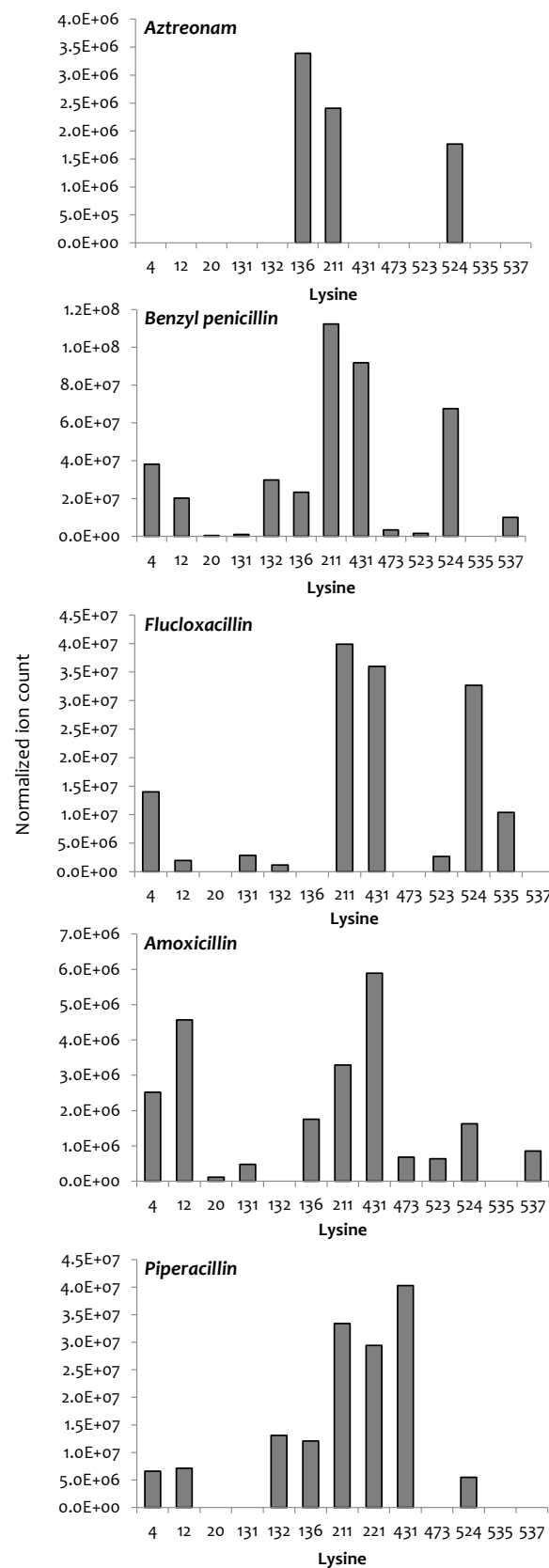


Figure 2. Epitope profile for piperacillin, amoxicillin, aztreonam, flucloxacillin and benzyl penicillin BSA adducts. Adducts were generated by culturing BSA with the drugs at a 1:50 ratio for 96 h. Proteins were denatured, digested with trypsin and desalted prior to characterization by mass spectrometry.

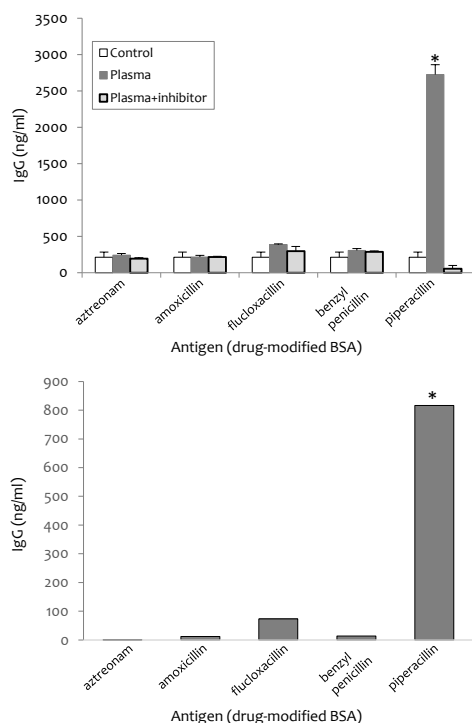


Figure 3. Detection of piperacillin IgG binding to different β -lactam BSA adducts using ELISA. (A) Results using plasma from the patient with highest levels of piperacillin-specific IgG is shown to illustrate control values and the effect of hapten inhibition. Data shows mean \pm SD hapten inhabitable antibody binding to the different BSA adducts. (B) Mean data showing hapten-inhabitable IgG values obtained with plasma from 4 piperacillin hypersensitive patients. Error bars have been omitted for clarity. Data was analysed with the Students T-test to compare differences between means.

Previous studies with PBMC and cloned T-cells from piperacillin hypersensitive patients revealed low levels of cross reactivity.^{4,5} Our recent discovery of piperacillin-specific IgG in hypersensitive patient plasma⁶ prompted us to investigate whether piperacillin-specific IgG bound to different β -lactam protein conjugates. Mass spectrometry revealed that the different drugs bound to a similar panel of lysine residues on the protein carrier BSA, albeit at different levels. However, the absence of piperacillin-specific IgG binding to amoxicillin, aztreonam and benzyl penicillin adducts and the low levels of binding seen with the flucloxacillin adduct shows that the humoral immune response induced in hypersensitive patients is also highly structurally specific. IgG antibodies must interact with the penicilloyl structure as well as the side chain of piperacillin to confer such specificity.

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ABBREVIATIONS

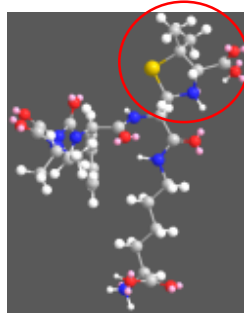
Bovine serum albumin (BSA), PBMC, peripheral blood mononuclear cells.

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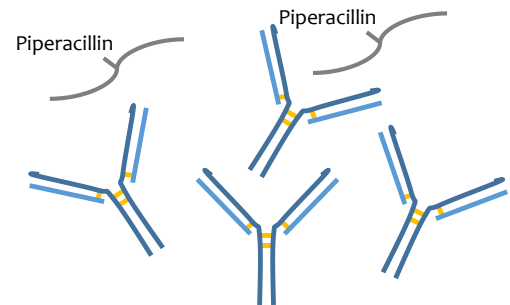
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Piperacillin hypersensitivity



Piperacillin-adducts formed at lysine residues



Anti-piperacillin IgG bind to adducts in a structurally-specific manner